The Preparation and Properties of Ficin Chemically Attached to Carboxymethylcellulose

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1. Purified ficin has been coupled to four CM-celluloses by reaction with their acid azide derivatives. Insoluble products containing 1.8–4.7 mg. of ficin/100 mg. of product and retaining 8.0–12.0% of the free enzyme's esterase activity have been obtained. 2. The amount of bound ficin in these preparations is dependent on the degree of carboxymethyl substitution of the CM-cellulose to which the ficin is attached. 3. A shift of the alkaline limb of the pH-activity curve of ficin when chemically attached to CM-cellulose has been shown. 4. Only a small loss has been observed in the enzymic activity of these products when stored at 2° for 4 months. They are more resistant than free enzyme to heat denaturation. 5. Columns of CM-cellulose-ficin have been packed. The degree of hydrolysis of perfused substrate has been measured for different flow rates through the column. 6. The properties of these derivatives have been discussed.

Water-insoluble derivatives of enzymes have been prepared in the following ways: (a) by chemical attachment of the enzyme to a reactive polymer (Bar-Eli & Katchalski, 1960, 1963; Cebra, Givol, Silman & Katchalski, 1961; Levin, Pecht, Goldstein & Katchalski, 1964; Mitz & Summaria, 1961; Manecke, 1962); (b) by physical adsorption of the enzyme to a charged polymer (Mitz, 1956; McLaren & Estermann, 1956; Barnett & Bull, 1959; Nikolaev & Mardashev, 1961; Nikolaev, 1962); (c) by entrapping the enzyme in the insoluble matrix of a cross-linked polymer (Bernfield & Wan, 1963); (d) by cross-linking of an enzyme by a bifunctional reagent (Quiocho & Richards, 1964).

The present paper describes the preparation and properties of ficin (EC 3.4.4.12) chemically attached to CM-celluloses. A preliminary account of some of this work has been given (Lilly, Money, Hornby & Crook, 1965).

MATERIALS AND METHODS

Purification of ficin. Crude ficin (250g.) (Cambrian Chemicals Ltd., London, S.E. 16) was purified by the method described by Hammond & Gutfreund (1959). A solution of the precipitate obtained from the first NaCl fractionation was exhaustively dialysed against deionized water and used in all subsequent experiments. The purified enzyme was assayed for esterase activity as described below. Protein

in solution was measured by the microbiuret method of Itzhaki & Gill (1964) and by the extinction at 280m μ . For this an exhaustively dialysed solution of ficin was freeze-dried and then further dried over P₂O₅. A solution of this material gave a specific extinction coefficient of 22.6.

Preparation of p-chloromercuribenzoate-blocked ficin. Ficin was reduced by cysteine (British Drug Houses Ltd., Poole, Dorset), separated from the latter on a column of Sephadex G-25 (Pharmacia Fine Chemicals Ltd., Uppsala, Sweden) and then blocked with PCMB* at pH 7.0 as described by Liener (1961). No residual activity towards BAEE was detected, but on treatment with cysteine 100% of the original activity could be recovered.

Preparation of CM-cellulose-ficin. By using the method of Mitz & Summaria (1961), ficin was coupled to CM-celluloses 30 and 70 (Whatman powder) and to CM-celluloses 50 and 90 (Mann Research Laboratories Inc., New York, N.Y., U.S.A.). These materials have different degrees of carboxymethyl substitution as shown in Table 1. The acid hydrazides of each CM-cellulose were prepared in 20g. quantities and stored in a desiccator. After conversion into their respective acid azides, 10g. of each material was suspended in 50 ml. of water at 1° and the suspension quickly titrated to pH 8.7. To each suspension 450 ml. of 0.05M-borate buffer, pH 8.7, was added, followed immediately by 5g. of the protein dissolved in 650 ml. of water. The suspensions were stirred for 60 min. at 1°, after which time the products were separated by filtration and washed

* Abbreviations: PCMB, *p*-chloromercuribenzoate; BAEE, *N*- α -benzoylarginine ethyl ester hydrochloride.

successively with two 11. quantities of mN-HCl and similar amounts of water. To ensure the removal of physically attached protein the products were further washed with 1.0M-NaCl ($2 \times 11.$), 0.5M-NaHCO_3 ($2 \times 500\text{ml.}$), 1.0M-NaCl ($11.$) and water ($2 \times 11.$). The products were resuspended in water and stored at 2° .

Determination of chemically attached protein. The protein content of the preparations was determined by hydrolysis in 6N-HCl for 42 hr. at 120° followed by estimation of the amino acids in the hydrolysate by the method of Cocking & Yemm (1954). A standard curve was obtained by treating known amounts of ficin in a similar manner.

Determination of enzymic activities. The esterase activity of ficin and CM-cellulose-ficin was determined by measuring the initial rate of hydrolysis of BAEE (L. Light and Co. Ltd., Colnbrook, Bucks.) at 25° with a TTT1c automatic titrator (Radiometer, Copenhagen, Denmark). Assays were carried out at pH 6.5 in a total reaction volume of 3.0 ml. in the presence of 5 mM-cysteine, mM-EDTA and 0.1 M-NaCl.

The proteolytic activity of the enzyme and its derivatives was determined by measuring the rate of hydrolysis of casein at 25° as described by Kunitz (1946).

For both assay methods the measured activity of CM-cellulose-ficin derivatives was found to be dependent on the rate of stirring of the reaction mixture. Therefore all assays were carried out at a stirring rate which produced maximal activity under the conditions of the assays.

Table 1. *Properties of commercial carboxymethyl-cellulose ion-exchange resins*

The degree of carboxymethyl substitution is expressed as the number of carboxymethyl groups/glucose monomer.

CM-cellulose	Exchange capacity (m-equiv./g.)	Degree of carboxymethyl substitution
30	0.3	0.049
50	0.5	0.083
70	0.7	0.118
90	0.9	0.154

RESULTS

Purification of ficin. A threefold purification of the crude ficin was observed in the final product. This material had V 5–6 μmoles of BAEE hydrolysed/min./mg. of protein and K_m $2.0 \times 10^{-2}\text{M}$. These results are in accordance with those reported by Holloway, Mathias & Rabin (1964) and Bernhard & Gutfreund (1956). The purified enzyme, when chromatographed at 2° on a CM-cellulose 70 column equilibrated with mM-acetate buffer, pH 4.4, was eluted by a sodium chloride gradient in a similar manner to that reported by Liener (1961).

Preparation of CM-cellulose-ficin. In the results given here all the bound protein is believed to be

Fig. 1. Relation between the exchange capacity of CM-cellulose and its protein-binding capacity. All preparations were carried out under identical conditions and the products were subjected to the same washing procedure. The protein content of each CM-cellulose-ficin was determined by the ninhydrin method. Experimental details are given in the text.

Table 2. *Effect of protein concentration in the coupling reaction mixture on the amount and enzymic activity of ficin bound by carboxymethylcellulose*

Experimental details are given in the text.

Prep. no.	Ficin (mg./g. of CM-cellulose-azide in coupling reaction)	Ficin coupled (mg./g. of derivative)	Esterase activity	
			(μmole of BAEE hydrolysed/min./mg. of prep.)	(μmole of BAEE hydrolysed/min./mg. of protein)
1	50	18	0.0135	0.80
2	100	26	0.019	0.73
3	200	30	0.021	0.69
4	300	37	0.022	0.60
5	400	43	0.025	0.58
6	500	47	0.025	0.54

chemically attached. The complete removal of the physically attached protein has been confirmed in a control experiment in which unmodified CM-cellulose replaced its corresponding acid azide in the coupling reaction mixture. In this case, when the product was subjected to the washing procedure described above, no residual protein and no esterase activity could be detected.

In coupling experiments with CM-cellulose 70 the amount of chemically attached protein was shown to be dependent on the reaction volume and the extent of the agitation. Therefore, in all comparative experiments, the coupling reactions were performed in identical vessels with the same degrees of agitation.

Fig. 2. Effect of the exchange capacity of the CM-cellulose on the activity of CM-cellulose-ficin preparations. The measurements of V (μ moles of BAEE hydrolysed/min./mg. of preparation) were made at 25° and pH 6.5 in the presence of 5 mM-cysteine, mM-EDTA and 0.1 M-NaCl. Experimental details are given in the text.

The effect of varying the amount of enzyme in the coupling reaction mixture, keeping all other conditions as described in the Materials and Methods section, was investigated with the acid azide of CM-cellulose 70. These results are shown in Table 2.

On reaction with identical amounts of enzyme under the same conditions the acid azides of four different CM-celluloses (see the Materials and Methods section) yielded products whose protein contents and enzymic activities were shown to be dependent on the degree of carboxymethyl substitution in the original cellulose (Figs. 1 and 2).

The effect of coupling PCMB-blocked ficin to the acid azide of CM-cellulose 70 was investigated. The amount of bound protein and its activity, after washing with cysteine, were found to be similar to those observed in a control experiment with unblocked enzyme (Table 3).

Properties of CM-cellulose-ficin. The effect of substrate concentration on the initial rates of hydrolysis of BAEE by CM-cellulose 70-ficin and CM-cellulose 90-ficin was investigated. Michaelis-Menten kinetics were shown to be obeyed and K_m 2×10^{-3} – 2.5×10^{-3} M was obtained for these two preparations by using the method of Lineweaver & Burk (1934) (Fig. 3). The esterase activities of preparations with CM-celluloses 30, 50, 70 and 90 were compared by assaying in a BAEE concentration that produced maximal initial velocities for each preparation. These results are given in Table 4.

The effect of pH on the initial rates of hydrolysis of BAEE by ficin and CM-cellulose-ficin preparations has been compared (Fig. 4). The displacement of the alkaline limb of the pH-activity curve with CM-cellulose 70-ficin has been observed to decrease on elevation of the ionic strength.

The measurement of the rates of hydrolysis of casein, at different pH values, by ficin and CM-cellulose 70-ficin revealed that there was no shift in the pH optimum when the enzyme was attached to the cellulose. It was therefore possible to compare

Table 3. *Coupling of ficin, pretreated with p-chloromercuribenzoate, with carboxymethylcellulose*

After coupling the PCMB was removed from the CM-cellulose-ficin by washing with cysteine. A control experiment was done with unblocked ficin. Experimental details are given in the text. Results for two separate experiments are shown.

Preparation	Protein content (mg. of protein/g. of prep.)	Esterase activity (μ mole of BAEE hydrolysed/min./mg. of prep.)
(a) With PCMB-ficin		
1	46	0.025
2	49	0.026
(b) With unblocked ficin		
1	48	0.023
2	45	0.027

the esterase and proteolytic activities of ficin and CM-cellulose-ficin derivatives at pH 7.0 as shown in Table 5.

Stability of CM-cellulose-ficin. The effect of temperature on the stability of CM-cellulose 70-ficin and ficin was investigated by incubation of the preparations at 66° for various times (Fig. 5) and at different temperatures for periods of 25 min. (Fig. 6). In both cases samples were rapidly cooled and assayed at 25°.

A suspension of CM-cellulose-ficin in water at pH 5.0 has been stored at 2° for up to 4 months with less than 15% decrease in activity. By assaying CM-cellulose 70-ficin and ficin over a period of several weeks, both in the presence and absence of cysteine, it was shown that the insoluble enzyme

was less dependent on cysteine for activation than the free enzyme (Table 6).

Preparations of CM-cellulose 30-ficin and CM-cellulose 70-ficin have been freeze-dried without any change in their enzymic activities and can be stored in this form.

Hydrolysis of BAEE by columns of CM-cellulose 70-ficin. Columns (3–5 cm. × 1.0 cm. diam.) of CM-cellulose 70-ficin have been packed and perfused with solutions of BAEE at different flow rates. At a substrate concentration of 0.3 mM BAEE in the perfusate, extents of hydrolysis of 19–99% have been observed (M. D. Lilly, W. E. Hornby & E. M. Crook, unpublished work) for flow rates in the range 15–0.5 ml./min.

DISCUSSION

Insoluble derivatives of ficin have been prepared by coupling the enzyme to CM-celluloses. In all

Fig. 3. Relation between substrate concentration and initial reaction velocities of the CM-cellulose 70-ficin- and CM-cellulose 90-ficin-catalysed hydrolysis of BAEE. ○, CM-cellulose 70-ficin; △, CM-cellulose 90-ficin. The initial reaction velocities (v) are expressed as μ moles of BAEE hydrolysed/min./mg. of preparation. Experimental details are given in the text.

Fig. 4. Effect of pH on the initial rate of hydrolysis of BAEE by ficin (○), CM-cellulose 70-ficin (△) and CM-cellulose 90-ficin (□). Measurements were made at 25° in the presence of 5 mM-cysteine, mM-EDTA and 0.1 M-NaCl. Experimental details are given in the text. Results were corrected for CO₂ uptake by carrying out blank assays in the absence of enzyme.

Table 4. *Esterase activity of ficin chemically attached to different carboxymethylcelluloses*

Experimental details are given in the text. Esterase activity coefficient is defined as the (maximal reaction velocity/mg. of protein) × 100/(maximal reaction velocity/mg. of free ficin).

Preparation	mg. of ficin/g. of derivative	Maximal esterase activity (V)		Esterase activity coefficient
		(μ mole of BAEE hydrolysed/min./mg. of prep.)	(μ moles of BAEE hydrolysed/min./mg. of protein)	
CM-cellulose 30-ficin	24.2	0.0097	0.400	8.0
CM-cellulose 50-ficin	32.6	0.0145	0.445	8.9
CM-cellulose 70-ficin	37.6	0.0220	0.585	11.7
CM-cellulose 90-ficin	42.3	0.0263	0.620	12.4
Ficin	—	—	5.00	100.0

Table 5. *Proteolytic activity of ficin chemically attached to carboxymethylcelluloses*

Experimental details are given in the text. Proteolytic activity coefficient is defined as the specific proteolytic activity of bound ficin $\times 100$ /specific proteolytic activity of free ficin.

Preparation	$\Delta E_{280m\mu}/\text{mg.}$ of prep.	$\Delta E_{280m\mu}/\text{mg.}$ of protein	Proteolytic activity coefficient	Esterase activity coefficient/ proteolytic activity coefficient
CM-cellulose 30-ficin	0.035	0.145	4.4	1.8
CM-cellulose 50-ficin	0.044	0.150	4.5	2.0
CM-cellulose 70-ficin	0.060	0.159	4.8	2.4
CM-cellulose 90-ficin	0.063	0.174	5.2	2.4
Ficin	—	3.3	100.0	1.0

Fig. 5. Effect of temperature on the stability of ficin (O) and CM-cellulose 70-ficin (Δ). The preparations were incubated at 66°, samples were withdrawn at intervals and cooled in an ice bath before being assayed at 25° for esterase activity towards BAEE. Experimental details are given in the text.

Fig. 6. Effect of different temperatures on the stability of ficin (O) and CM-cellulose 70-ficin (Δ). After incubation of the preparations at the different temperatures for 25 min., samples were removed, cooled in an ice bath and assayed at 25° for esterase activity towards BAEE. Experimental details are given in the text.

Table 6. *Comparison of activation of free ficin and bound ficin by cysteine*

A CM-cellulose 70-ficin suspension and a ficin solution were stored at 2°. Samples were taken at intervals and assayed for esterase activity in the presence of 5 mM-cysteine and without cysteine. Experimental details are given in the text. Activities are expressed as a percentage of the original activity.

Time (days)	Ficin activity		CM-cellulose 70-ficin activity	
	With cysteine	Without cysteine	With cysteine	Without cysteine
7	100	76	100	100
14	90	50	100	100
21	66	10	100	100

cases the protein contents of our derivatives are less than those reported by Mitz & Summari (1961). for their insoluble derivatives. However, we have shown that the washing procedures adopted by these authors did not remove all physically attached ficin. Similar observations have been made with chymotrypsin by C. Money & E. M. Crook (unpublished work).

The bound ficin in these derivatives exhibited 9–12% of its original esterase activity and 4–5% of its proteolytic activity. The results obtained on coupling PCMB-blocked ficin to CM-cellulose 70-azide show that this loss in activity is not due to the formation of a thioester between the reactive thiol group of ficin and the azide group on the cellulose. In all insoluble derivatives of ficin the loss of proteolytic activity was greater than the loss of esterase activity when compared with free ficin. The greater activity of the derivatives towards smaller substrate molecules suggests some form of steric hindrance on the part of the cellulose. Such interference by the cellulose could also hinder diffusion of substrates and products to and from the active site of the enzyme. This could account for the observation that increased rates of agitation of reaction mixtures produced higher rates of

hydrolysis of casein and BAEE by CM-cellulose-ficin derivatives.

Another reason for loss in enzymic activity could be that the chemically attached enzyme is bound to the CM-cellulose in some conformation with less freedom of movement than that of the free enzyme. Such a restriction would reduce denaturation phenomena in the insoluble enzyme and an effect of this kind has been observed for the thermal denaturation of the derivatives when compared with ficin.

Values of 2.0×10^{-3} – 2.5×10^{-3} M for the apparent K_m values of ficin derivatives on CM-cellulose 70 and 90 are 10–12% of that observed for the free enzyme. Goldstein, Levin & Katchalski (1964) have reported a decrease in the K_m of trypsin when it is attached to a copolymer of maleic acid and ethylene. These authors have ascribed this result to the presence of areas of increased substrate concentration around the polymer due to electrostatic attraction between the positively charged substrate and the carboxyl groups of the polymer. As our system is qualitatively similar the decrease in the K_m of ficin after coupling to CM-cellulose can be explained in this way. The shift observed in the pH-activity curve of insoluble ficin is due probably to a similar phenomenon in which the carboxyl groups of the CM-cellulose produce an environment around the enzyme in which the concentration of protons is higher than that measured in the overall suspension. This explanation has been used by McLaren & Estermann (1957) to account for a similar shift in the pH-activity curve of chymotrypsin adsorbed on kaolinite and by Goldstein *et al.* (1964) for shifts observed in trypsin chemically attached to the copolymer of maleic acid and ethylene.

Although there is a loss in the enzymic activity of the bound protein in these derivatives of ficin, nevertheless, they have many practical advantages. Their increased stability, greater resistance to denaturation and reduced susceptibility to autolysis makes them suitable for use over long periods of time. By virtue of their insolubility they can be recovered from reaction media quickly and

efficiently without contamination of substrate and products. With material packed in columns we have shown that it is possible to exercise great control over the rate and extent of conversion of substrate into products.

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